The Role of Calcium in the Regulation of Vascular Smooth Muscle Alpha Actin Gene Expression

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by

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The heart is a dynamic organ that is made up of multiple cell types including muscle and non-muscle. In general the heart is capable of changing due to many factors including development, physiological response, and pathological conditions. Fibrotic (scarring) and hypertrophic (increase in cell size) diseases of the heart are often associated with messengers (such as calcium (Ca^{2+})) and pathways that activate proteins normally expressed only in the developing heart. One of these proteins, vascular smooth muscle alpha actin (SMA), is the predominant actin in smooth muscle, but is not normally expressed in the adult heart. However, SMA is activated in response to heart transplant and the associated rejection process (fetal reactivation). The focus of this project is to determine the role of Ca^{2+} in the regulation of SMA in resident non-muscle cell types of the heart.

By using cultured cells we have determined that Ca^{2+} levels and/or Ca^{2+} agonists effect previously identified transcription factors that bind to the SMA promoter. In addition, regulation of at least one of these transcription factors, YB-1, in non-muscle cells may be controlled by its relative concentration within the nucleus and cytoplasm. A better understanding of the transcriptional factors regulated by Ca^{2+} and those associated with fetal gene reactivation will give a better understanding of SMA and its relation to cardiac disease.

Introduction

Tissue fibrosis is the hallmark of many human diseases. In general, it is associated with the altered structure and compromised function of many organs damaged by a wide range of injuries or agents including infection, autoimmune processes, chemical insults, etc. While many of the causes remain unknown, fibrotic diseases are characterized by non-cellular material (scar tissue) accumulating in affected organs. In the transplanted heart, it often precedes hypertrophy (cell growth), another indicator of compromised function, which can lead to overload and eventual failure. One important cell type critically important in fibrotic tissue remodeling and scar formation is the myofibroblast.

Myofibroblasts are derived from tissue fibroblasts that are "activated" when they acquire the ability to express muscle (myo-) proteins involved in both contraction and cellular migration. Myofibroblasts have been shown to provide the contractile function necessary for closing open wounds and may play a similar role in reorganizing damaged tissue (Also see Cogan, *et al*., 2002; Subramanian *et al*., 2002). For many years, our lab has been studying the genetic mechanisms responsible for myofibroblast activation. One of the key components is transforming growth factor-ß (TGF-ß), a pleiotropic cytokine involved in both immune suppression and alterations in cellular growth and differentiation. We have demonstrated that TGF-ß activates the smooth muscle actin (SMA) gene, an indicator of fibrosis that is also reactivated in cardiac myocytes during rejection. SMA "reactivation" is thought to occur through two distinct pathways. The first pathway affects transcription by causing TGF-ß responsive Smad proteins to translocate to the cell nucleus (Also see Massague, *et al*., 2000). The second pathway

involves activation of a protein called Erk, which affects transcription through the transcription factor EGR-1.

We have attempted here to decipher the potential role that calcium plays in the regulation of SMA by TGF-ß in fibroblasts, particularly in regard to the two pathways mentioned above. Because increases in intracellular calcium have been shown to be a factor in cardiomyocyte hypertrophy (Wilkins, *et al*., 2002), we have also attempted to understand whether the link between fibrosis and hypertrophy in the heart is related to alterations in cellular calcium. More specifically, the focus of this thesis is to identify factors associated with SMA transcription, whose interplay may be affected by calcium levels. It is our hope that after understanding the mechanisms that regulate this important indicator gene, we may begin to develop methods that may be useful in the treatment of many types of heart diseases.

Materials and Methods

Cell Culture Methods – Mouse AKR-2B embryonic fibroblasts were maintained in McCoy's 5A medium (Cambrex Bio Science Walkersville, Walkersville, MD) supplemented with 5% heat-inactivated fetal bovine serum (hiFBS) and penicillin-streptomycin (Invitrogen, Carlsbad, CA). Nonhuman primate Cos7 kidney fibroblasts were maintained in DMEM (4.5 g/l D-glucose) supplemented with penicillin-streptomycin and 10% hiFBS. All cell lines were grown in a humidified incubator at 37ºC with 5% CO_2. Fibroblasts were rendered quiescent by a 24-hr exposure to HEPES-buffered DMEM (1.0 g/l D-glucose) containing 0.5% hiFBS, and penicillin-streptomycin-Fungizone. Ionomycin (1μg/ml, final concentration), a calcium ionophore, was added to

select cultures as a pretreatment for 4hrs. Recombinant human TGF-ß1 (5 ng/ml final concentration; R&D Systems, Minneapolis, MN) was then added to cultures for 6hrs. After treatment, RNA or protein extracts were collected for measurements. (Also see Subramanian *et al*., 2004)

Preparation of cDNA and Real Time PCR Measurements

RNA was extracted from the cells using TRIzol reagent (1ml) and chloroform (200 µl). After vortexing the mixture and centrifuging for 15 min at 14,000 rpm, the upper aqueous layer was collected. Equal volume of isopropanol was added to precipitate RNA and collected by centrifuging for 10 min at 14,000 rpm. The RNA pellet was then washed with 75% ethanol, centrifuged for 10 min at 14,000 rpm, and dissolved in 30 µl DEPC-treated water. The RNA concentration was determined by a spectrophotometer and 1 µg of RNA was used for each reverse transcription to first-strand complementary deoxyribonucleic acid (cDNA). The cDNA synthesis was performed by combining 1 µg of RNA with RNA/primer mixtures and by following the procedure described in SuperScript™ First-Strand Synthesis System for RT-PCR by the manufacturer (Invitrogen, Carlsbad, CA). The cDNA was used in real-time polymerase chain reaction (PCR) to measure the amount of mRNA initially present in cells. Amplification of the target cDNA was done by using the TaqMan (Applied Biosystems, Foster City, CA) fluorescent probe and by following the procedure given by Applied Biosystems for their real-time PCR machine (Applied Biosystems, Foster City, CA).

Preparation of Protein Extracts

Cells were washed twice with Dulbecco's phosphate-buffered saline (PBS), scraped into fresh PBS, centrifuged at 3000 rpm, and resuspended in eight packed-cell volumes of

hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.5 mM dithiothreitol [DTT]). Cells were allowed to swell for 10 min on ice before transfer to a Dounce homogenizer and disruption with it. Nuclei were collected from ruptured cells by centrifuging for 15 min at 4000 rpm and suspended in one-half packed pellet volume of ice-cold, low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). High salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) equal to one-half packed pellet volume, was added and the nuclei extracted with gentle rocking for 20 min at 4ºC. Extracted nuclear proteins were collected by centrifuging for 20 min at 14,000 rpm removing the supernatant, and dialyzing it against 50 volumes of dialysis buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT. After dialysis, supernatants were clarified by centrifuging at 14,500 rpm for 20 min for use in biochemical assays. (Also see Subramanian *et al*., 2004)

DNA-binding Assay (DBA)

Synthetic oligonucleotide probes used during this study correspond to sequences present in the mouse SMA 5'-flanking region (Min *et al*., 1990). Experiment mixtures containing nuclear extract (50 μg of protein) and biotinylated oligonucleotides (100pmol; Integrated DNA Technologies, Coralville, IA) were incubated in reaction mixtures containing poly(dI-dC), 10 mM Tris, pH 7.5, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 0.12mM PMSF, 4% glycerol. Protein:biotin:DNA complexes were captured on streptavidin-immobilized paramagnetic particles (Promega, Madison, WI; 0.6 ml/reaction, 30 min incubation). After washing four times with buffer containing 25 mM

Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl, bound protein was eluted using 2X protein denaturing buffer and analyzed by SDS-PAGE and immunoblotting procedures. (Also see Subramanian *et al*., 2004)

Mammalian Protein Overexpression Plasmids, Cell Transfection, and Reporter Gene Assays

Fibroblasts at 40-50% confluence were transfected with the SMA promoter:reporter fusion plasmid VSMP4 and/or plasmids, encoding various transcriptional regulatory proteins by using Mirus (Invitrogen, San Diego, CA) transfection reagent, and by following protocol provided by the manufacturer (Also see Cogan *et al*., 2002). Plasmids encoding human Smad 2/3/4 proteins were kindly provided by Drs. L. Choy and R. Derynck (University of California San Francisco, San Francisco, CA). Plasmids were purified using QIAGEN preparative resin and a protocol provided by the manufacturer (QIAGEN, Valencia, CA). Forty-eight hours after transfection, cells were washed with PBS and then lysed using chloramphenicol acetyltransferase (CAT) enzyme-linked immunosorbent assay (ELISA) lysis buffer (Roche Applied Science, Indianapolis, IN). Whole cell extracts were clarified at 14,000 rpm for 10 min at 4ºC and stored at -20ºC before assay. Total protein in extracts was determined by bicinchoninic acid colorimetric assay (Pierce Chemical, Rockford, IL). Equivalent amounts of lysed protein were evaluated by immunoblot to verify protein overexpression. CAT reporter gene activity was determined using a commercial ELISA kit (Roche Applied Science). Reporter gene expression was normalized with respect to total cell protein, and transefections were routinely performed in triplicate and repeated three to five times. Data sets were

subjected to analysis of variance to assess statistical significance set at $p < 0.05$. (Also see Subramanian *et al*., 2004)

Immunoblotting

Proteins (10- μg aliquots) were size fractionated by SDS-PAGE by using 10% polyacrylamide gels and then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). After overnight blocking at 4ºC in Tris-buffered saline (TBS; 25 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% (wt/vol) nonfat dry milk and 0.5% bovine serum albumin, blots were incubated with selected rabbit or goat polyclonal antibodies (2 μg/ml) for 90 min at room temperature with gentle rocking. Antibodies specific for Sp1, EGR, and Smad proteins were obtained commercially (Santa Cruz Biotechnology, Santa Cruz, CA) and two different Pur protein-specific antibodies (anti-Purα 291-313 and anti-Purβ 302-324) plus a pan-specific Purα/β (42-69) were described previously (Kelm *et al*., 1999a; Subramanian *et al*., 2002). Blots were washed four times at room temperature over a 40-min period in TBS containing Tween 20 (0.05% vol/vol). Horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (1:1500) or horseradish peroxidase-conjugated donkey anti-goat secondary antibody (1:2000) was applied to respective blots for 60 min after which time the blots were washed, as described above, and developed for chemiluminescence (ECL; Amersham Bio-sciences, Piscataway, NJ). (Also see Subramanian *et al*., 2004)

Results

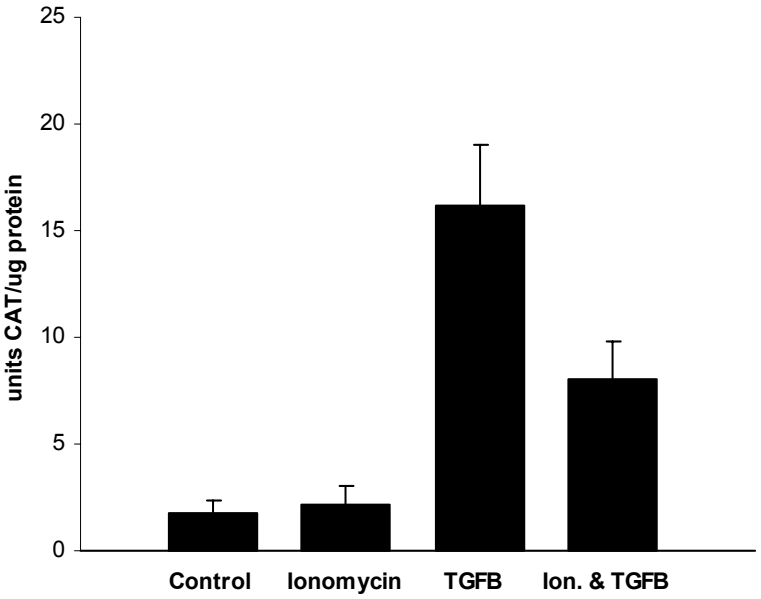

Fig 1. AKR cells were transfected with the SMP4 plasmid and treated with ionomycin and TGFB. CAT Elisa measured reporter gene activty. Ionomycin eliminated some TGFB activation but it did not effect basal acivity of the SMP4 reporter plasmid.

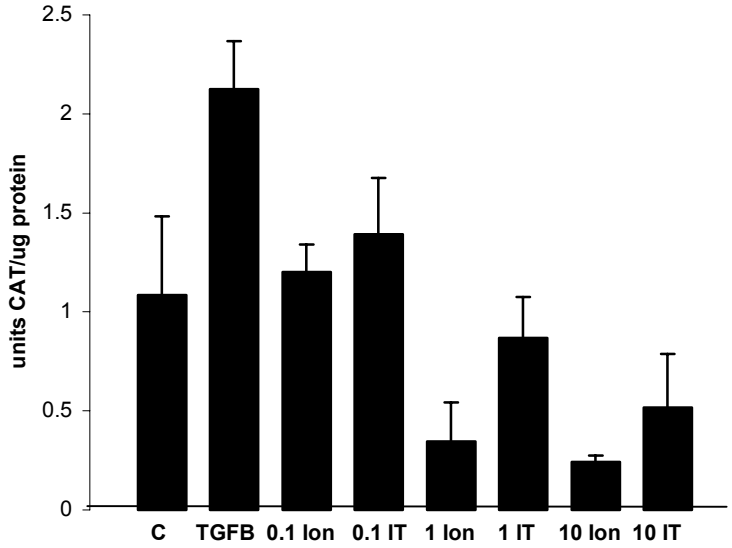

Fig 2. AKR cells were transfected with the SMP4 plasmid and treated with TGFB and differing concentrations of ionomycin. CAT Elisa measured reporter gene activty. The optimal ionomycin level to use for future experiments is 1 µg/ml since it eliminated both basal activity and TGFB activation.

In order to determine the role that calcium plays in the regulation of the SMA gene in fibroblasts we first tested the effect of the calcium ionophore ionomycin on the SMA reporter plasmid (SMP4) with and without TGF-ß, a known inducer of SMA transcription. Fig. 1 shows that ionomycin by itself did not affect SMP4 activity but it did reduce TGF-ß activation by a factor of two. A t-test confirmed a significant difference between TGF-ß and TGF-ß with ionomycin. ($t = 6.2$, $df = 3$, $p = 0.05$)

Given the result, we varied concentrations of ionomycin in order to determine an experimental optimum concentration for future experiments. Fig. 2 shows CAT assays using levels of 0.1, 1, and 10 µg/ml of ionomycin with and without TGF-ß. Results from this study indicate the optimal level

was 1 µg/ml because it eliminated TGF-ß activation and had no outward effects of cell structure or viability. 0.1 µg/ml did not significantly affect TGF-ß activation. At 10 µg/ml, inhibition of TGF-ß appeared to reach a maximum. However, a significant effect on cell survival and viability was noted (visual observation, not shown). A t-test confirmed a significant difference between TGF-ß and TGF-ß with 1 µg/ml of ionomycin. ($t = 9.7$, $df = 3$, $p = 0.05$)

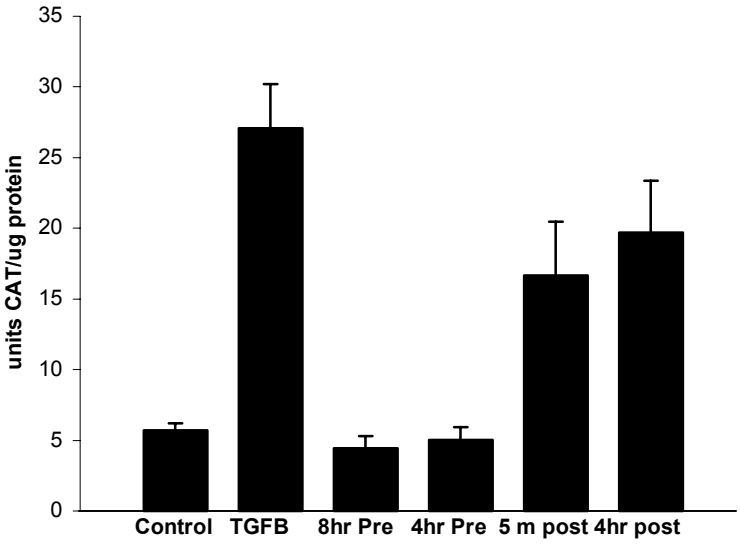

Fig 3. AKR cells were transfected with the SMP4 plasmid and treated with ionomycin and TGFB for differing times. CAT Elisa measured reporter gene activty. The 8 & 4hr pretreatment with ionomycin eliminated TGFB activation. Therefore, the 4hr pretreatment was selected as optimal time.

Using the optimal 1 µg/ml ionomycin concentration discovered in the preceding experiment, a temporal study was performed varying the amount of time transfected cells were treated with the drug prior to or following TGF-ß activation. 8 hr and 4 hr pretreatments along with 5 min and 4 hr post treatments relative to TGF-ß addition were performed and reporter gene activity was assayed. Fig. 3 demonstrates that pretreatment for 8hr and 4hr were similar, because both eliminated TGF-ß activation of the SMP4 reporter plasmid. In contrast, post treatments were shown to have little effect on TGF-ß activation. A t-test confirmed a significant difference between TGF-ß and TGF-ß with 4hr pretreatment of ionomycin. ($t = 18.8$, $df = 3$, $p =$

0.05) For this reason, subsequent studies using ionomycin were standardized using 4 hr pretreatment with the 1 μg/ml optimized dosage.

Because reporter gene studies indicated changes in *de vovo* transcription from the SMA promoter in response to calcium, experiments were performed next to determine if the calcium ionophore, ionomycin, affects mRNA levels derived from the native SMA gene. Using pre-determined optimal concentrations and the prescribed pretreatments determined above, mRNA levels were compared to controls using quantitative real-time PCR assays. Fig. 4 shows that consistent with the results obtained in reporter gene assays, pretreatment with ionomycin eliminated TGF-ß activity, but had no effect on basal transcription of SMA. A t-test confirmed a significant difference between TGF-ß and TGF-ß with ionomycin SMA mRNA amounts. ($t = 4.4$, $df = 3$, $p = 0.05$)

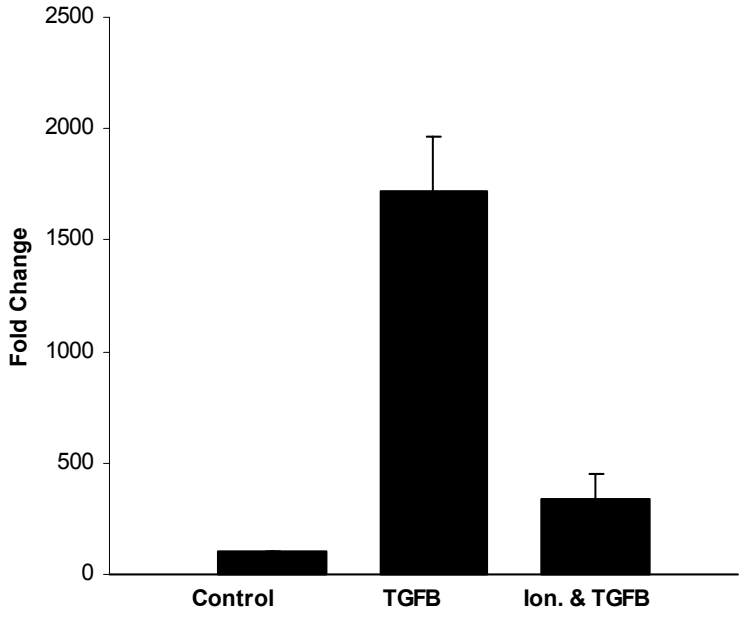

Fig 4. AKR cells were treated with ionomycin and TGFB. Real-time PCR measured mRNA levels. Pretreatment with ionomycin eliminated TGFB induction but did not effect basal transcription.

Inhibition of TGF-ß inducibility by ionomycin may act by affecting transcription factors within the cell that regulate SMA expression. In order to determine the effect that TGF-ß alone or TGF-ß and ionomycinon had on the nuclear level or promoter binding level of proteins known to bind the SMA promoter (Model 1), DNA binding assays (DBA) were performed. Fig. 5 shows that TGF-ß caused an increase in binding of EGR to the SMA promoter, while ionomycin eliminated this increase. All other transcription factors examined,including Pur alpha and SP1 were unchanged by either drug. This result, suggests that cellular calcium levels may affect pathways of TGF-ß induction, which result in the decreased binding of EGR-1 to the SMA promoter.

Model 1. Sequence of double stranded SPUR probe used to evaluate dynamic interplay of SMA enhancer-binding proteins.

GAAGCGAGTGGGAGGGGATCAGAGCAAGGGGC

GGA or AGG: potential Pur and Sp1 binding sites

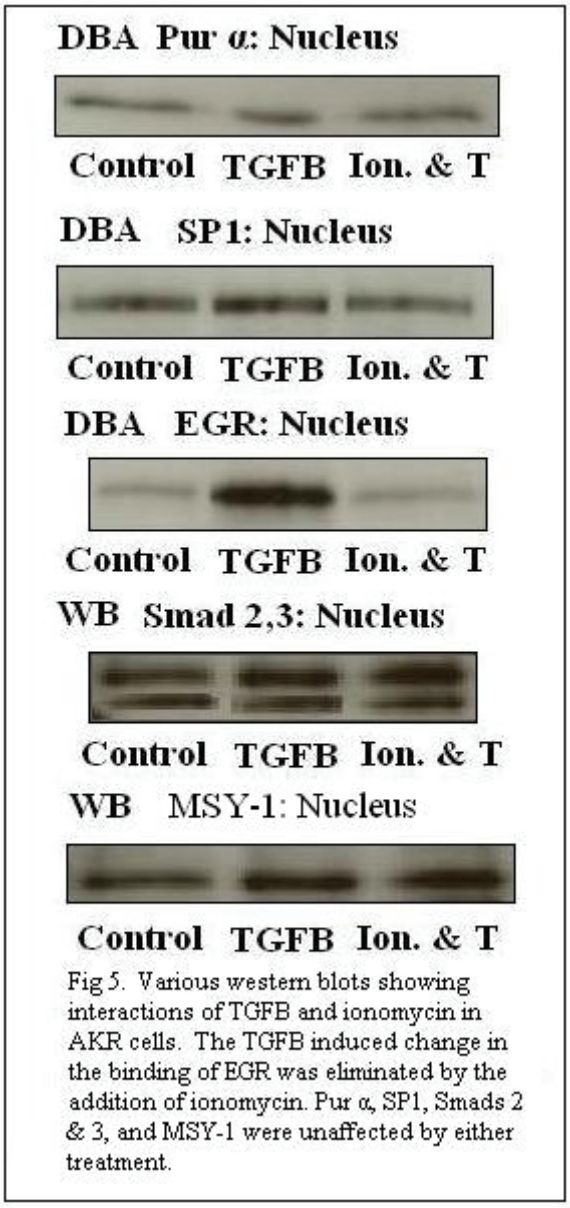

Fig 5. Various western blots showing interactions of TGFB and ionomycin in AKR cells. The TGFB induced change in the binding of EGR was eliminated by the addition of ionomycin. Pur α, SP1, Smads 2 & 3, and MSY-1 were unaffected by either treatment.

Previous studies in our lab and elsewhere indicate that TGF-ß activation can occur through two separate pathways. One of these pathways, the ERK pathway can affect the activity of the transcription factor EGR-1, shown above to also be regulatable by calcium. The other pathway involves the nuclear translocation of Smad proteins and, in our system, is also affected by the single-stranded repressor binding proteins Pur alpha/beta and MSY-1 (Subramanian *et al*., 2004). To

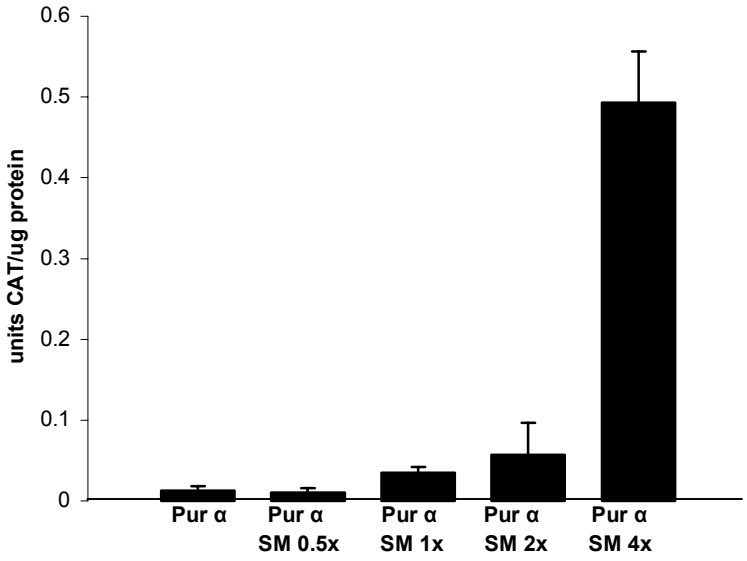

Fig 6. Cos7 cells were transfected with Smad proteins and Pur α. CAT Elisa measured reporter gene activty. A high enough concentration of Smad proteins are present they can overcome Pur α repression, with a 4x concentration of Smads being the ideal concentation.

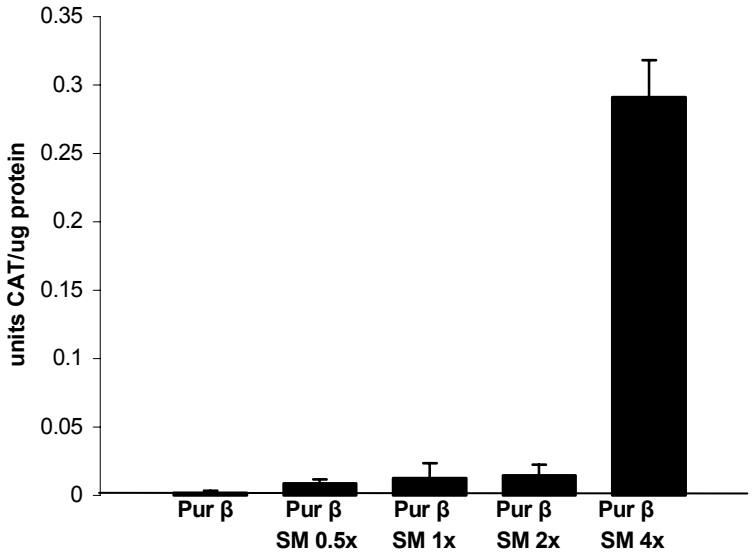

Fig 7. Cos7 cells were transfected with Smad proteins and Pur β. CAT Elisa measured reporter gene activty. A high enough concentration of Smad proteins can over come Pur β repression, with a 4x concentration of Smads being the best concentration.

examine more closely the stoichiometric requirements of these transcription factors in our cell culture model, we over-expressed Smad proteins (Smad 2,3,4) at increasing concentrations and examined the effects on SMA reporter gene activity. Fig 6 shows that increasing amounts of Smad protein relative to fixed concentrations of the Pur alpha repressor are required for overriding SMA repression. Activation of SMA in the presence of Pur alpha was shown to be highest when an equimolar amounts of Smad DNA and Pur alpha DNA was transfected, suggesting a 1:1 stoichiometric ratio in overriding repression. A t-test confirmed a significant difference between Pur alpha and Pur alpha with equimolar amounts of Smad DNA. ($t = 23.8$, $df = 3$, $p = 0.05$)

Similarly, Fig. 7 shows that the effects of increasing concentrations of Smad

levels on Pur ß, another known repressor, and optimal relief of repression was seen at stoichiometric ratios of transfected DNA at 1:1 ratios. A t-test confirmed a significant difference between Pur ß and Pur ß with equimolar amounts of Smad DNA. ($t = 7.8$, $df = 3$, $p = 0.05$) The results of both experiments indicate the increasing levels of Smad proteins may override repressor function, although absolute expression remains lower in cells co-transfected with Pur beta.

It is possible that activator and repressor functions are competitive, because Fig. 8 shows that in the absence of overexpressed repressors, activation increases with increased Smad overexpressoin activity. A t-test confirmed a significant difference between no Smads and maximum Smad levels. ($t = 95.7$, $df = 3$, $p = 0.05$) It is also possible that Smad levels cause changes in protein-protein interactions that affect activator/repressor availability in the nucleus or on the promoter.

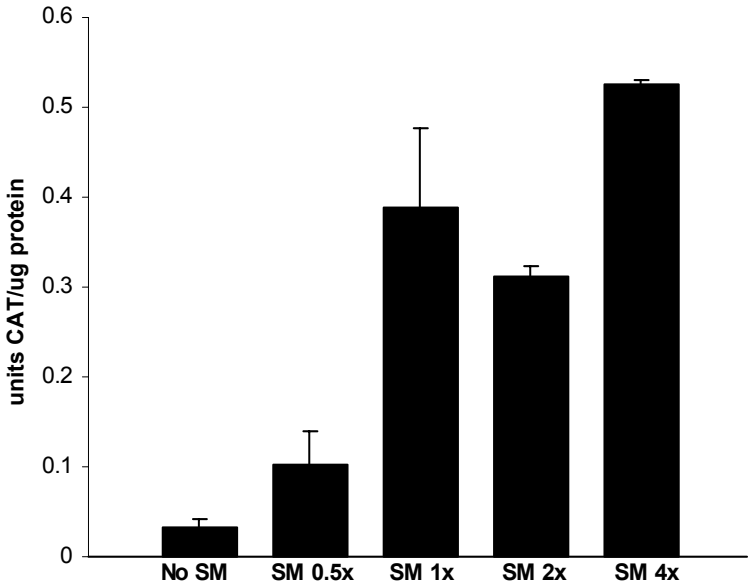

Fig 8. Differing concentration of Smad proteins were transfected into Cos7 cells and used to look for the best concentration to cause activation. CAT Elisa measured reporter gene activty. The optimal concentration of Smad proteins to use for future experiments is the 4x.

Using the maximum levels of Smads overexpression determined above in Cos7 cells, a time point experiment was performed to determine the onset of activation following transfection. Fig. 9 illustrates that after 48 hrs, Smad overexpression had the greatest effect on activation of the SMA reporter gene. A t-test confirmed a significant difference between zero hours and 48 hours. ($t = 16.8$, $df = 3$, $p = 0.05$)

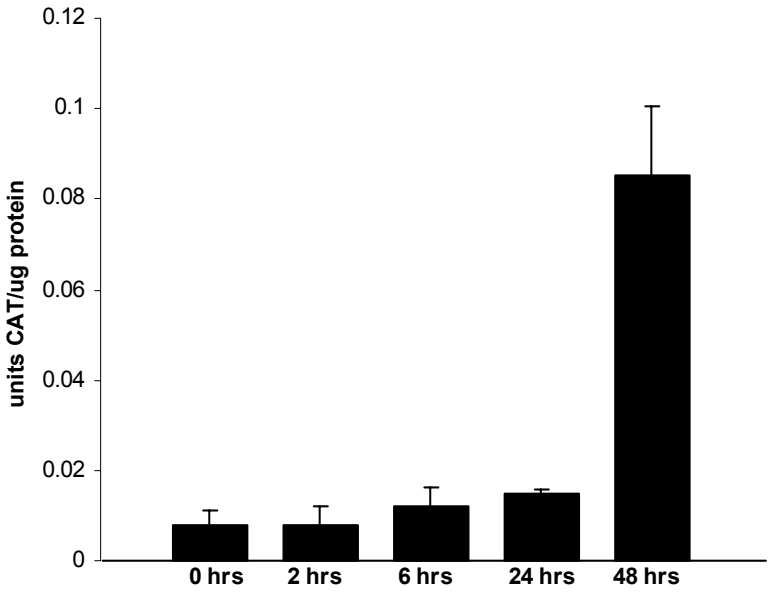

Fig 9. 4x Smad proteins were transfected into Cos7 cells and harvested at differing time points. CAT Elisa measured reporter gene activity. After 48 hrs, the Smad proteins show the greatest activation on SMP4 reporter plasmid.

Western blot assay performed with nuclear extracts from cells transfected for corresponding time points showed that Smad proteins also reach peak concentrations in the nucleus after 48 hrs following transfection, Fig 10. Increases in nuclear Smad levels after 48 hour transfection were not seen to affect the nuclear levels of either native Pur alpha or Pur beta or of any known transcriptional activators of the SMA gene in transfected cells. (data not shown) However, the levels of YB-1, a single-stranded DNA binding

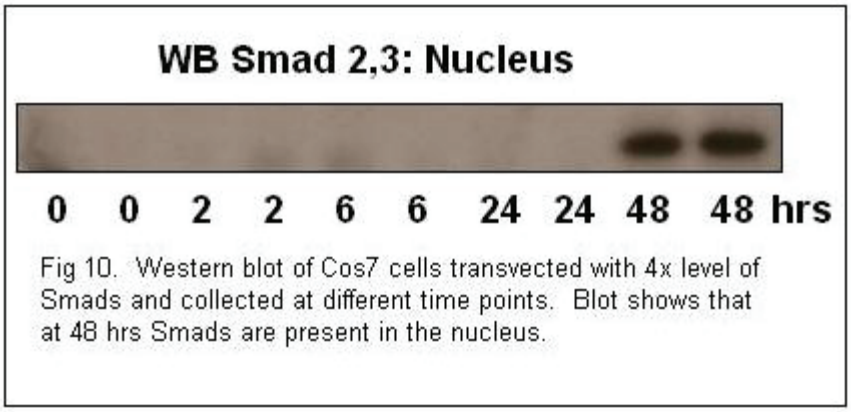

Fig 10. Western blot of Cos7 cells transvected with 4x level of Smads and collected at different time points. Blot shows that at 48 hrs Smads are present in the nucleus.

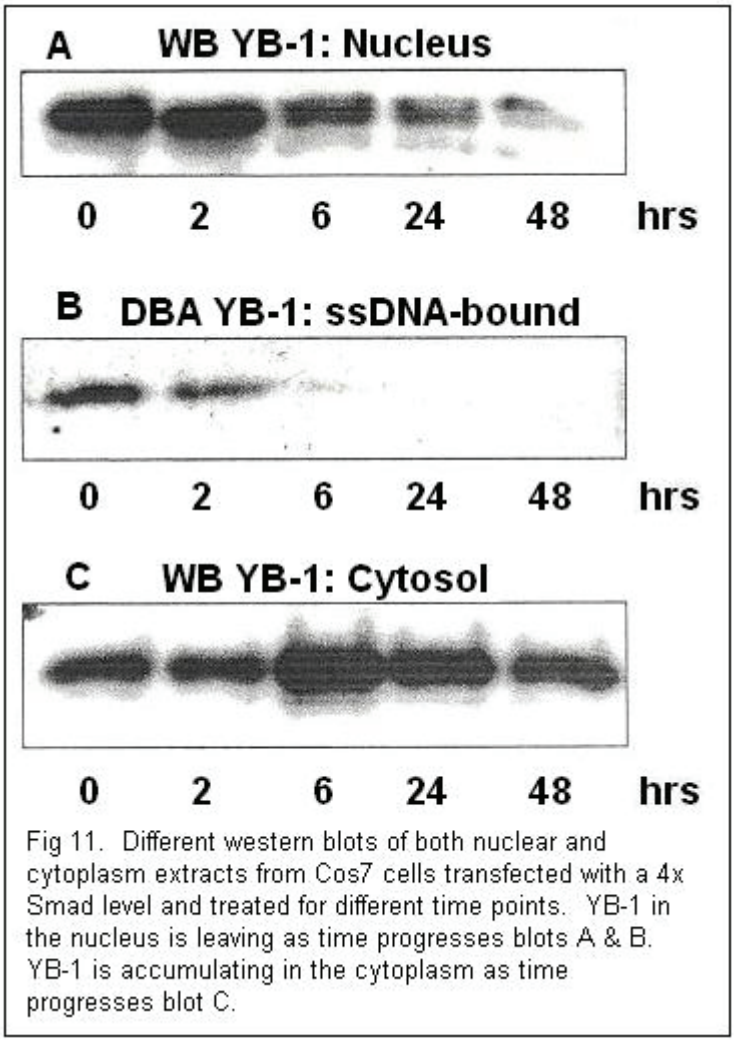

Fig 11. Different western blots of both nuclear and cytoplasm extracts from Cos7 cells transfected with a 4x Smad level and treated for different time points. YB-1 in the nucleus is leaving as time progresses blots A & B. YB-1 is accumulating in the cytoplasm as time progresses blot C.

protein with a similar repressor function on the SMA promoter as Pur alpha and Pur ß dropped corresponding to rises in Smad nuclear levels. Fig. 11 shows that increased Smad overexpression and its accumulation in the nucleus affects YB-1 binding to a SMA promoter single-stranded probe as well as its nuclear and cytoplasmic levels. More specifically, as Smad levels rise in transfected nuclei, YB-1, a repressor of SMA gene transcription, decreases in availablility by presumably leaving the nucleus and accumulating in the cytoplasm.

Discussion

A primary purpose of this study was to identify factors associated with SMA transcription, whose interplay may be affected by calcium levels. In the presence of the calcium ionophore, ionomycin, TGF-ß activated transcription of SMA was highly reduced or eliminated as measured by both reporter gene analysis and real-time PCR assays. This data suggests that Ca^{2+} levels within the cell seem to play a role, particularly in the TGF-ß inducible regulation of SMA gene. Previous studies in our lab support this finding and suggest that transcriptional mechanisms responsible for basal and inducible expression of this important marker gene may rely on distinct sets of pathways and

transcription factors. (Also see Cogan, *et al*., 2002) Since expression of SMA is a key step in the formation of the myofibroblast phenotype, these findings may also have important implications in the wound healing process and in fibrotic diseases of a variety of organs.

Inhibition of both native SMA mRNA and SMA truncated reporter gene levels suggest that ionomycin^{+} eliminates TGF-ß activation through a cis regulatory element and trans-acting protein whose activity is modified by calcium. Two potential candidate proteins, Pur α and YB-1 (known as MSY-1 in mouse), both repressors of SMA (Kelm, *et al*., 1999b), were unaffected (Fig. 5) by treatment with either TGF-ß alone or TGF-ß and ionomycin, in this study. The only protein whose level and promoter binding activity was affected was EGR-1. EGR-1 binding to the SMA gene was highly inducible by TGF-ß but was eliminated in the presence of ionomycin. EGR-1 has been shown to bind to the SMA promoter at a site important for TGF-ß inducibility, a site which also binds to the transcription factors SP-1 and Pur alpha (unpublished). Further studies will be required to determine whether the binding of EGR-1 to this critical regulatory element is important for TGF-ß induction and whether calcium levels within the cell alter this induction through EGR-1. Because the ERK pathway, a protein kinase pathway involved in TGF-ß induction, is known to regulated EGR-1, the involvement of calcium in this pathway will be an important focus of future studies,

Smad proteins are involved in TGF-ß signal transduction from the membrane to the nucleus and can act independently from the ERK pathway. In this study, however, we detected no effect on nuclear Smad levels by either TGF-ß or ionomycin treatment. These results suggest that Ca^{2+} does not elecit changes in nuclear localization under the

conditions looked at in this study. Because nuclear localization of Smad proteins following TGF-ß induction is well established and has been shown to be measurable only at short time intervals (<2 hours in our previous studies), it is possible that the conditions necessary for detecting these changes were inadequate. Despite the fact that no changes in nuclear Smad levels were seen when cells were treated with TGF-ß or TGF-ß and ionomycin, our previous studies have indicated that Smads can influence Pur alpha and YB-1 action on the SMA promoter. Results obtained here illustrate that high concentrations of Smad proteins transfected and over-expressed in Cos7 cells, reverse the effects of Pur alpha and Pur beta repression on SMA transcription. Smad overexpression relieved repression most effectively when transfected at an equimolar ratio to Pur alpha and Pur Beta (Smad 4x level). Consistent with these findings, SMA activity was highest in studies where Smad protein levels were maximally expressed and appeared in the nucleus. In addition, an increase in nuclear Smad levels was coincident with decreased levels of YB-1 protein (human homologue to MSY-1). This gradual decrease, presumably a result of Smad nuclear localization, was also accompanied by an accumulation of YB-1 in the cytoplasm over the same time period (as measured by interaction between cytoplasmic extracts and the Pyr-rich ssDNA MSY- binding site probe). Future studies will be aimed at determining how and why the accumulation of Smad proteins in the nucleus result in YB-1 shuttling from nucleus to cytoplasm and whether this is necessary and or important for Smad-dependent gene activation.

In summary, Ca^{2+} eliminates TGF-ß activation and may affect how certain transcription factors, particularly EGR-1, bind to the SMA promoter. The antagonistic effect of calcium on TGF-ß activation, will require further study, particularly considering the fact

that increases in cellular calcium have been linked to hypertropy (Wilkins, *et al.*, 2002). One possible model, consistent with our data, is that the calcium flux is necessary for controlling the action of the fibrotic cytokine TGF-ß, may lead to hypertrophic growth in chronically stimulated cells. In our study, we found no clear evidence that Ca^{2+} affects the TGF-ß responsive Smad proteins or other transcription factors (such as YB-1 or MSY-1, Pur alpha, Sp1) previously implicated in TGF-ß activation of SMA. However, alterations in Smad protein levels within the nucleus were shown here to be concamittent with YB-1 translocation from nucleus to cytoplasm. This translocation event may represent a dynamic process important for regulating inducible expression of the SMA gene., and, therefore, may be an important marker for fibrotic disease. Whether or not this process is linked to calcium is unknown and will require further studies.

Acknowledgements

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